XXXIV. THE EXISTENCE IN THE BILE OF AN INHIBITOR FOR HEPATIC ESTERASE, AND ITS NATURE.

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(Received March 23rd, 1920.)

It is generally accepted that the addition of bile to pancreatic juice accelerates its lipolytic activity. As is well known, there can be extracted from the liver an enzyme capable of rapidly hydrolysing the lower esters, and the object of the present research was to discover whether a similar effect would be produced on this esterase by the addition of bile.

The research was commenced in 1914 at the suggestion of Prof. Cathcart, and the greater part of the work was done at that time. I was then unaware of Loevenhart's [1906, 1, 2] investigations on the effect of bile-salts on the liver esterase. My work had to be left uncompleted, and it was not until its resumption at the end of the past year that Loevenhart's paper on the above subject came to my notice.

Various methods of preparing the liver extract were tried, the first attempts being directed towards obtaining a dry preparation of liver by extraction with acetone and ether, or alcohol and ether. Such dry preparations were found, however, to possess very slight esterolytic properties (Exp. 1). This is in accordance with the experiences of Loevenhart [1906, 2, p. 433].

Exp. No. 1. Cat's Liver.

Quantities used:		Minutes	Cc. N/10 NaOH
		5	0.7
Ethyl butyrate	0.25 cc.	10	1.1
Extract	0.5 g.	15	1.3
Water	5.0 cc.	20	1.5
Phenolphthalein	4 drops	25	1.6
		30	1.6

The method of obtaining a dry active preparation described by Loevenhart [1906, 2, p. 432], which is a modification of a method cited by Magnus [1904], was not tried. Ramond's [1904] ether extraction method was found to be unsatisfactory.

10 % aqueous extracts were found to be quite satisfactory for the work in view, and were generally used. 5 g. of liver were ground in a mortar with a suitable amount of sand and water, and the extract made up to 50 cc. with water. The whole was then strained through muslin into a stoppered bottle and the bottle well shaken before pipetting off any quantity for use.

Such extracts were found to be highly active, and, despite the fact that, even by thorough shaking it would seem impossible to obtain a homogeneous mixture, successive quantities of equal amount withdrawn from the bottle were found to have practically the same esterolytic activity (Exps. 2 and 3). If a small amount of toluene were added to the extract, the diminution in its activity with age was remarkably slow, as shown in Exp. 4. Throughout the work, however, fresh extracts were used for each experiment.

Exp. No. 2. Cat's Liver. 10 % Extract.

Quantities in each tube:		Minutes	Cc. N/10 NaOH	
			First 5 cc.	Second 5 cc.
Ethyl butyrate	0.5 cc.	5	1.4	1.4
Extract	5.0 cc.	10	2.5	2.6
Water	5.0 cc.	15	3.6	3.5
Phenolphthalein	4 drops	20	4.6	4.5
		25	5.3	5.4
		30	6.1	6.0

Exp. No. 3. Cat's Liver. 10 % Extract.

Quantities:		Minutes	Cc. N/10 NaOH	
			First 5 cc.	Second 5 cc.
As in No. 2		5	2.3	2.2
		10	4.3	4.2
		15	6.0	5.9
		20	7.6	7.5
		25	8.9	8.7
		30	9.9	9.8

Exp. No. 4. Cat's Liver. 10 % Extract.

Quantities:		Minutes	Cc. N/10 NaOH	
			14th Dec.	22nd Dec.
As in No. 2		5	1.3	1.4
		10	2.9	2.6
		15	4.5	3.8
		20	5.6	4.6
		25	6.7	5.2
		30	7.7	5.7

Ethyl butyrate was the ester used in all cases, and, as the butyric acid set free rapidly destroyed the hepatic enzyme, the following method which, to a certain extent, obviated this, and also, it was thought, gave a closer approximation to the *in vivo* conditions, was adopted.

Tubes containing the ester, indicator (phenolphthalein), and water were placed in a water-bath at 38° for five minutes. The extract was then added and the contents of the tubes exactly neutralised with N/10 NaOH. Having been replaced in the water-bath, a burette was fixed above each containing N/10 NaOH, and titrations of the acidity developed were carried out every five minutes during a period of half-an-hour. Under such conditions esterolysis was found to be much more active than when titration of the mixture was performed only at the end of the period of incubation.

Exp. No. 9. Rabbit's Liver. 10 % Extract.

	Minutes	Cc. N/10 NaOH	
		Without bile	With bile
Quantities:	5	0.5	0.3
As in No. 5	10	1.0	0.45
	15	1.35	0.65
	20	1.75	0.85
	25	2.1	1.0
	30	2.45	1.15

Exp. No. 10. Rabbit's Liver. 10 % Extract.

	Minutes	Cc. N/10 NaOH	
		Without bile	With bile
Quantities:	5	0.7	0.65
As in No. 5	10	1.3	1.1
	15	1.8	1.5
	20	2.3	1.9
	25	2.7	2.2
	30	3.1	2.5

Exp. No. 11. Rabbit's Liver. 10 % Extract.

	Minutes	Cc. N/10 NaOH	
		Without bile	With bile
Quantities:	5	0.9	0.2
As in No. 5 except that	10	1.75	0.2
0.5 cc. bile used	15	2.3	0.2
	20	2.9	0.2
	25	3.2	0.3
	30	3.6	0.3

Exp. No. 12. Rabbit's Liver. 10 % Extract.

	Minutes	Cc. N/10 NaOH	
		Without bile	With bile
Quantities:	5	0.9	0.2
As in No. 11	10	1.5	0.3
	15	1.95	0.3
	20	2.5	0.4
	25	2.9	0.4
	30	3.3	0.4

Exp. No. 13. Guinea-pig's Liver. 10 % Extract.

	Minutes	Cc. N/50 NaOH	
		Without bile	With bile
Quantities:	5	1.8	1.2
As in No. 5	10	3.0	1.9
N/50 NaOH used for titration	15	3.7	2.6
	20	4.2	3.2
	25	4.5	3.5
	30	4.8	3.7

That the dilution due to the addition of the bile has a negligible effect on the reaction is shown in Exp. No. 14.

Exp. No. 14. Cat's Liver. 10 % Extract.

	Minutes	Cc. N/10 NaOH	
		Without water	With 0.15 cc. water
Quantities:	5	1.45	1.4
As in No. 5	10	2.55	2.55
	15	3.45	3.4
	20	4.3	4.3
	25	5.05	5.0
	30	5.75	5.65

Control tubes containing ester without extract, and extract without ester, treated in an exactly similar manner, developed in half-an-hour an acidity of comparatively negligible amount.

B. IS THE INHIBITING SUBSTANCE SPECIFIC FOR EACH SPECIES?

Exps. Nos. 15 to 21 were carried out in an endeavour to find whether the liver extract of one animal would be inhibited by the bile of another animal of a different species, and if the inhibition by the foreign bile would be as marked as that produced by the animal's own bile.

*Exp. No. 15. Livers from Cat, Dog, and Rabbit. 10 % Extracts.***A. Cat's Liver Extract. Quantities:—As in No. 5.**

Minutes	Cc. N/10 NaOH			
	Without bile	With cat's bile	With dog's bile	With rabbit's bile
5	0.35	0.25	0.25	0.35
10	0.55	0.35	0.35	0.5
15	0.65	0.35	0.35	0.6
20	0.8	0.35	0.4	0.7
25	0.85	0.4	0.5	0.8
30	0.9	0.45	0.5	0.8

B. Dog's Liver Extract. Quantities:—As in No. 5.

Minutes	Cc. N/10 NaOH			
	Without bile	With dog's bile	With cat's bile	With rabbit's bile
5	0.65	0.4	0.5	0.5
10	1.0	0.45	0.65	0.7
15	1.15	0.45	0.7	0.85
20	1.25	0.5	0.75	0.9
25	1.35	0.5	0.75	0.9
30	1.4	0.5	0.85	1.0

C. Rabbit's Liver Extract. Quantities:—As in No. 5.

Minutes	Cc. N/10 NaOH			
	Without bile	With rabbit's bile	With dog's bile	With cat's bile
5	1.1	0.65	0.75	0.85
10	2.2	1.05	1.3	1.6
15	3.15	1.4	1.7	2.3
20	3.9	1.65	2.1	2.8
25	4.5	1.85	2.2	3.4
30	5.1	2.0	2.3	3.9

Exp. No. 16. Cat's Liver Extract. Quantities:—As in No. 2. Bile 0.15 cc.

Minutes	Cc. N/10 NaOH			
	Without bile	With cat's bile	With rabbit's bile	With dog's bile
5	3.9	1.6	2.0	2.6
10	8.1	3.0	4.4	5.7
15	12.0	4.5	6.9	8.8
20	15.4	5.8	9.7	12.0
25	18.4	7.1	12.1	15.1
30	20.9	8.2	14.4	17.6

Exp. No. 17. Cat's Liver. 10 % Extract. Quantities:—As in No. 16.

Minutes	Cc. N/10 NaOH		
	Without bile	With cat's bile	With ox bile
5	1.7	1.2	1.2
10	3.2	2.2	2.3
15	4.7	3.1	3.3
20	6.0	3.5	4.0
25	7.0	3.8	4.3
30	7.9	4.1	4.8

Exp. No. 18. Rabbit's Liver. 20 % Extract. Quantities:—As in No. 16.

Minutes	Cc. N/10 NaOH		
	Without bile	With rabbit's bile	With ox bile
5	2.4	1.6	1.6
10	5.5	2.8	3.4
15	8.5	4.0	5.0
20	10.9	5.05	6.4
25	13.5	6.1	7.8
30	15.7	6.9	9.0

Exp. No. 19. Cat's Liver. 10 % Extract. Quantities:—As in No. 16.

Minutes	Cc. N/10 NaOH		
	Without bile	With cat's bile	With ox bile
5	1.0	1.1	0.75
10	1.9	2.2	1.7
15	3.0	3.2	2.7
20	4.2	4.3	3.7
25	5.3	5.0	4.6
30	5.9	5.3	5.3

Exp. No. 20. Cat's Liver. 10 % Extract. Quantities:—As in No. 16.

Minutes	Cc. N/10 NaOH		
	Without bile	With cat's bile	With ox bile
5	2.3	1.7	1.9
10	4.3	3.7	3.8
15	6.0	5.4	5.3
20	7.6	6.9	6.3
25	8.9	8.0	7.4
30	9.9	9.0	8.4

Exp. No. 21. Livers from Dog and Cat. 10 % Extracts.

A. Dog's Liver Extract. Quantities:—As in No. 5.

Minutes	Cc. N/10 NaOH		
	Without bile	With dog's bile	With cat's bile
5	0.7	0.4	0.7
10	1.2	0.5	1.1
15	1.5	0.55	1.3
20	1.7	0.6	1.4
25	1.8	0.6	1.5
30	1.95	0.65	1.55

B. Cat's Liver Extract. Quantities:—As in No. 5.

Minutes	Cc. N/10 NaOH		
	Without bile	With cat's bile	With dog's bile
5	0.3	0.25	0.2
10	0.45	0.4	0.2
15	0.6	0.5	0.3
20	0.7	0.6	0.35
25	0.8	0.65	0.35
30	0.85	0.65	0.4

C. THE NATURE OF THE INHIBITOR.

It has been shown by various workers [von Fürth and Schütz, 1906; Hewlett, 1905; Loevenhart and Souder, 1906; and others] that a solution of bile-salts will produce an acceleration of the hydrolysis of both fats and the lower esters by pancreatic juice, similar to that obtained on adding the whole bile. Further it has been demonstrated by Magnus [1906] that bile-salts synthetically prepared have the same action. The same author showed in a previous paper [1904] how the esterolytic ferment of the liver could be rendered inactive by dialysis, and that the dialysable portion was thermostable, soluble in alcohol but insoluble in ether, and destroyed by incineration. Magnus used as zymolyte amyl salicylate and his results were confirmed by Loevenhart [1906, 1, 2], who further discovered that the activity of the non-dialysable portion could be restored by the addition of bile-salts.

Loevenhart found, however, that while this was true of the hydrolysis of amyl salicylate, ethyl butyrate behaved in a different manner. Dialysis did not cause the liver extract to lose its property of hydrolysing the latter ester; and when bile-salts were added to a non-dialysed liver extract its activity towards ethyl butyrate was diminished.

The following experiments were performed without knowledge of this part of Loevenhart's work and are fully confirmatory of his results.

Exps. 22 to 24 were carried out to find if the inhibitory substance were dialysable. A quantity of bile was dialysed and the effect on the esterase of both dialysate and residue determined (Nos. 22 and 23). Part of the dialysate was again dialysed. The effect of this double dialysate is shown in No. 24.

Exp. No. 22. Dog's Liver. 10 % Extract. Quantities:—As in No. 2.

Minutes	Cc. N/10 NaOH		
	Normal	Dialysate added	Residue added
5	1.5	1.4	1.2
10	2.7	2.0	1.8
15	3.2	2.6	2.1
20	3.9	3.0	2.5
25	4.2	3.3	2.8
30	4.6	3.7	3.1

Exp. No. 23. Cat's Liver. 10 % Extract. Quantities:—As in No. 2.

Minutes	Cc. N/10 NaOH		
	Normal	Dialysate added	Residue added
5	2.1	1.9	2.2
10	4.5	4.1	4.1
15	7.0	6.1	6.0
20	9.0	7.8	7.6
25	10.6	9.2	9.2
30	12.0	10.6	10.7

Exp. No. 24. Cat's Liver. 10 % Extract. Quantities:—As in No. 2.

Minutes	Cc. N/10 NaOH	
	Normal	Double dialysate added
5	2.2	2.2
10	4.5	4.4
15	6.8	6.5
20	8.7	8.2
25	10.5	9.5
30	12.0	10.8

It is evident that the inhibitory substance is dialysable, though the residue from dialysis still shows considerable inhibitory power. In the light of subsequent work it would seem that the bile was not allowed to dialyse for a sufficient length of time.

An alcoholic extract of the bile was made, the extract evaporated to dryness, and the material so obtained tested for inhibitory power. Exp. No. 25 shows the marked inhibition obtained on adding a small quantity of this dried alcoholic extract.

Exp. No. 25. Rabbit's Liver. 20 % Extract. Quantities:—As in No. 2.

Minutes	Cc. N/10 NaOH	
	Normal	Alcoholic extract added
5	2.4	1.4
10	5.3	2.0
15	8.5	2.8
20	11.1	3.3
25	13.5	3.8
30	15.7	4.4

Exps. Nos. 26 to 30 show the inhibition obtained on adding progressively smaller quantities of the evaporated alcoholic extract (A.E.). In No. 30 only 1 mg. of the material was used. In these only the difference between the acidity of the normal tube and the tube with alcoholic extract added (*i.e.* the amount of inhibition expressed in cc. *N*/10 NaOH) is given.

Cat's Liver. 10 % Extract.

Minutes	Inhibition in cc. <i>N</i> /10 NaOH				
	Exp. 26 ca. 10 mg. A.E.	Exp. 27 ca. 8 mg. A.E.	Exp. 28 ca. 6 mg. A.E.	Exp. 29 ca. 4 mg. A.E.	Exp. 30 1 mg. A.E.
5	0.3	0.4	0.3	0.0	0.0
10	0.6	0.7	0.5	0.0	0.2
15	1.0	1.0	0.6	0.1	0.2
20	1.1	1.2	0.6	0.1	0.2
25	1.4	1.2	0.8	0.4	0.2
30	1.4	1.3	0.9	0.5	0.1

That the alcohol-soluble portion of the bile contains all the inhibitory substance, none remaining in the residue, is shown in Nos. 31 and 32.

Exps. Nos. 31 and 32. Cat's Liver. 10 % Extract. Quantities:—As in No. 2.

Minutes	Cc. <i>N</i> /10 NaOH		Cc. <i>N</i> /10 NaOH	
	Normal	Residue from alcoholic extraction	Normal	Residue from alcoholic extraction
5	1.8	1.9	1.9	2.0
10	4.0	4.0	4.1	3.9
15	6.2	6.1	6.3	6.2
20	8.0	8.0	8.1	8.0
25	9.8	9.7	10.0	9.9
30	11.3	11.2	11.5	11.5

A solution in water of the dried alcoholic extract was then treated with ether. The precipitate was dried and the filtrate evaporated to dryness. Solutions in water of the material from each were investigated. The inhibitory substance was evidently insoluble in ether (No. 33).

Exp. No. 33. Cat's Liver. 10 % Extract. Quantities:—As in No. 2.

Minutes	Cc. <i>N</i> /10 NaOH		
	Normal	Filtrate	Residue
5	2.5	2.6	1.0
10	5.3	5.8	1.9
15	7.4	7.8	2.5
20	9.3	9.6	3.2
25	11.5	11.8	3.7
30	13.7	14.1	4.2

Exps. Nos. 34 and 35 show the inhibition obtained, using a 3 % aqueous solution of Plattner's crystals, while No. 36 shows that the inhibitory power is not diminished but rather increased when the bile-salts are purified by recrystallisation.

Exps. Nos. 34 and 35. Cat's Liver. 10 % Extract. Quantities:—As in No. 2.

Minutes	Cc. N/10 NaOH		Cc. N/10 NaOH	
	Normal	0.15 cc. bile-salt solution added	Normal	0.15 cc. bile-salt solution added
5	1.4	1.1	1.4	1.1
10	2.5	2.0	2.6	2.0
15	3.6	2.8	3.5	2.8
20	4.6	3.4	4.5	3.6
25	5.3	3.8	5.4	4.5
30	6.1	4.2	6.0	5.0

Exp. No. 36. Cat's Liver. 10 % Extract. Quantities:—As in No. 2.

Minutes	Cc. N/10 NaOH		
	Normal	Before recrystallisation	After recrystallisation
5	1.6	1.2	1.3
10	2.8	2.4	2.3
15	4.3	3.5	3.4
20	5.5	4.5	4.4
25	6.8	5.6	5.4
30	7.8	6.4	6.2

The sensitiveness of the hydrolysis of ethyl butyrate to the inhibiting action of bile-salts was found to be very considerable (Nos. 37 to 43, amounts of inhibition in cc. N/10 NaOH only given). In this connection Loevenhart [1906, 2] found that concentrations of 0.02 % and under, accelerated rather than inhibited the reaction. I have been unable to confirm this, finding inhibition to be produced in concentrations as low as 0.005 %. Concentrations lower than this were not tried, as it was thought that the method of estimating the activity of the enzyme was not sufficiently accurate to allow of this being done.

Exps. Nos. 37 to 43. Cat's Liver. 10 % Extract. Quantities:—As in No. 2.

Minutes	Inhibition caused by bile-salts in concentration of:						
	2 %	1 %	0.5 %	0.1 %	0.05 %	0.01 %	0.005 %
5	0.8	0.5	0.4	0.2	0.1	0.0	0.0
10	1.5	1.6	1.1	0.3	0.2	0.0	0.1
15	2.5	2.6	1.7	0.8	0.5	0.1	0.4
20	3.5	3.5	2.7	1.1	0.7	0.3	0.4
25	4.4	4.2	3.4	1.6	1.0	0.6	0.6
30	5.2	5.4	4.1	2.4	1.2	0.8	0.7

D. OTHER INHIBITORS.

Rosenheim and Shaw-McKenzie [1910] found that sodium cholate, saponin, and serum, among other substances, acted as accelerators to the pancreatic lipase in its action on olive oil. The presence of cholesterol inhibited this acceleration. Von Fürth and Schütz [1906] also showed that sodium cholate was an active accelerator of the hydrolysis of fat by pancreatic juice. The activation of pancreatic lipase by saponin was investigated by Flohr [1919],

who showed that the acceleration produced was proportional to the lowering of surface-tension caused by the saponin. In the case of ricin lipase, however, saponin caused inhibition, and this, he concluded, was due to a poisoning action on the enzyme.

Exp. No. 44 shows the effect of cholalic acid and its derivatives, choleic and deoxycholeic acids. These substances were preparations made and kindly supplied by Dr S. R. Schryver. The first two produced inhibition, the latter, acceleration.

Exp. No. 44. Cat's Liver. 10 % Extract. Quantities:—As in No. 2.

Minutes	Cc. N/10 NaOH			
	Normal	Cholalic acid	Choleic acid	Deoxycholeic acid
5	2.6	1.8	2.4	2.6
10	5.6	3.8	4.8	5.8
15	8.3	5.6	6.4	8.8
20	10.7	7.5	8.1	12.0
25	13.0	9.2	9.6	15.9
30	15.1	10.8	11.1	19.4

That the sensitiveness of the reaction to cholalic acid was as great as to the bile-salts is shown by Exps. 46 to 52 (amount of inhibition in cc. N/10 NaOH only given).

Exps. Nos. 45 to 51. Cat's Liver. 10 % Extract. Quantities:—As in No. 2.

Minutes	Inhibition caused by cholalic acid in concentration of:						
	2 %	1 %	0.5 %	0.1 %	0.05 %	0.01 %	0.005 %
5	0.2	0.0	0.0	0.0	0.1	0.0	0.0
10	0.3	0.4	0.2	0.1	0.2	0.0	0.0
15	1.1	1.0	0.7	0.6	0.3	0.2	0.2
20	1.7	1.4	1.4	1.0	0.6	0.4	0.3
25	2.6	2.0	1.6	1.4	0.7	0.8	0.4
30	3.3	2.6	2.1	1.7	0.9	1.1	0.6

The effect of saponin was investigated and found to produce slight acceleration in large concentration (3 %, Exp. 52); in weaker concentrations (0.5 %, Exp. 53) little change was noted.

Exps. Nos. 52 and 53. Rabbit's Liver. 10 % Extract. Quantities:—As in No. 2.

Minutes	Cc. N/10 NaOH		Cc. N/10 NaOH	
	Normal	Saponin (3 %)	Normal	Saponin (0.5 %)
5	2.6	2.8	2.6	2.7
10	5.8	6.2	5.3	5.2
15	8.8	9.7	8.4	8.4
20	12.1	13.0	11.5	11.4
25	15.1	16.3	14.8	14.6
30	17.9	19.4	17.7	17.4

Cholesterol showed no marked activity in either direction (Nos. 54 and 55).

Exps. Nos. 54 and 55. Cat's Liver. 10 % Extract. Quantities:—As in No. 2.

Minutes	Cc. N/10 NaOH		Cc. N/10 NaOH	
	Normal	Cholesterol	Normal	Cholesterol
5	2.1	2.2	1.5	1.6
10	4.5	4.7	2.9	3.0
15	7.0	7.1	4.4	4.6
20	9.0	8.9	5.5	5.8
25	10.6	10.5	6.5	7.0
30	12.0	11.9	7.4	8.1

SUMMARY AND CONCLUSIONS.

1. There has been found to be present as a constant constituent in the bile of all the animals examined, a substance possessing the power of inhibiting the hepatic esterase.

2. Investigation of the action, on the esterase of one animal, of the bile of other species, showed that the inhibitor is probably of the same nature in all biles. At the same time, the results suggest that the concentration of the inhibitor in the bile of a particular animal is that best adapted for producing inhibition of the esterase in that animal.

3. Chemically the inhibiting substance was found to be soluble in alcohol, but insoluble in ether. A solution of Plattner's crystals was found to be as active as the bile in producing inhibition, and, since purification of the crystals by recrystallisation led to no loss of inhibitory power, the inhibitor may be concluded to be the bile-salts.

4. Esterolysis by the hepatic enzyme is extremely sensitive to the presence of bile-salts, as low a concentration as 0.005 % producing inhibition. Since the reaction is also inhibited by cholalic acid and is equally sensitive to this substance, it may be concluded that the bile-salts act as inhibitors by reason of the cholalic acid portion of their molecule.

5. The inhibition does not appear to be in any way due to the physical properties of bile-salts in lowering surface-tension as saponin does not produce a similar result.

6. Cholesterol does not inhibit the action of the esterase.

In conclusion I should like to record my great indebtedness to Prof. E. P. Cathcart for much help and guidance in the prosecution of the research.

Part of this work was done during the tenure of a part-time Carnegie Scholarship.

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